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Molecular systematics and diagnosis☆

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Abstract

This collection of articles provides an account of six presentations delivered at the 19th International Conference of the World Association for the Advancement of Veterinary Parasitology (WAAVP) (held in New Orleans, Louisiana, USA, from 10 to 14 August 2003) in a symposium session on Molecular Systematics and Diagnosis, organised and chaired by R.B. Gasser and D.S. Zarlenga. The focus was on recent advances in molecular tools for specific and genotypic identification, diagnosis, systematics and population genetics, with special emphasis on investigations of parasitic nematodes and protists.

Keywords: Diagnosis; Molecular taxonomy; Phylogeny; Population genetics; *Trichinella*; Cyathostomins; Filarioids; Hookworms; Lungworms; Tissue-forming coccidia; *Wohlbachia*; Zoonoses; Ribosomal DNA; Mitochondrial genome

Introduction—advances in the diagnosis and systematics of parasites of veterinary importance: new and exciting prospects

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The control of parasitic diseases of veterinary importance depends on the rapid and accurate detection of the aetiological agents, as well as the ability to characterise parasites on the basis of epidemiologically useful features. These include host specificity, public health significance in terms of zoonotic potential, virulence and drug sensitivity. Traditional diagnostic techniques involving microscopy have thus been complemented by a variety of molecular tools. The application of such tools has also helped to resolve taxonomic issues which resulted in much controversy in the past, when new species or 'strains' were described on the basis of phenotypic characteristics and/or epidemiological observations in particular endemic areas. A formal nomenclature is essential for effective communication and provides the stability which underpins epidemiological investigations. However, the lack of morphological differences between such 'variants' only compounded an often confusing 'taxonomic picture' which has, in many cases, taken decades to resolve. Such was the situation with *Echinococcus*, *Trichinella*, the cyathostomins and other nematodes as well as the coccidia discussed in this Symposium, and many other parasites of veterinary importance. As a result of the application of molecular tools, many taxonomic issues have been resolved and, as a consequence, communication has been markedly enhanced at all levels—from the classroom to the field. In addition, ongoing molecular epidemiological investigations on a variety of parasitic diseases are providing predictive data of immense value for control.

The application of molecular biological methods to parasite taxonomy and epidemiology has much to do with the detection and analysis of genetic variation within and among parasites. In recent years, the focus has been on defining appropriate regions of DNA to use for detecting variation at different taxonomic levels. This requires choosing molecular tools capable of discriminating genetic variants at different hierarchical levels, and the region of DNA examined must be appropriate to the level of questions being addressed (Table 1). Characterising such genetic variation is also dependent upon appropriate and rigorous analysis (Constantine, 2003), the reliability of which is enhanced when a number of different genetic loci is used. The value of such molecular tools is greatest if they can be applied directly to faecal or tissue specimens, as well as environmental samples and food, and if there is the potential to automate such procedures. In this respect, PCR-based techniques have provided veterinary parasitologists with very powerful epidemiological tools. Thus, if the appropriate molecular tools can be combined with PCR, then the major limiting factors for parasite characterisation of obtaining sufficient quantities of parasite material and a particular life cycle stage can be obviated. The remaining challenge of field applicability is likely to be overcome in the near future with advances in both sampling protocols and storage of parasite isolates.

The development of appropriate PCR-based procedures has been of great value for socio-economically important parasites, such as *Cryptosporidium*. PCR-based procedures have largely circumvented the problem of obtaining only tiny amounts of parasite material by allowing the direct characterisation of parasite isolates from faecal or environmental samples. Although a rationalisation of the number of recognised species of *Cryptosporidium* took place in the early 1990s (see O'Donoghue, 1995), molecular characterisation is resulting in the re-naming of several species (Mosier and Oberst, 2000; Fayer et al., 2001). A number of genetic loci has proved particularly useful for studies of *Cryptosporidium*, and their application in different laboratories to a range of isolates has served to support the list of species currently recognised, many of which are morphologically identical

Table 1
Molecular tools for the characterization of genetic diversity in parasites at different hierarchical levels

Function[a]	Purpose	Regions of DNA
Discrimination above level of species	Phylogeny	Highly conserved coding regions: e.g., small subunit of ribosomal DNA, features of mitochondrial DNA
Discrimination between species	Taxonomy/diagnosis/Epidemiology	Moderately conserved regions: e.g., coding genes in mitochondrial DNA, internal transcribed spacers of ribosomal DNA
Discrimination between intraspecific variants/strains/genotypes	Population genetics/breeding systems/host adapted strains/conservation	Variable regions: e.g., allozyme, random amplification of polymorphic DNA, amplified fragment length polymorphism, pulse field gel electrophoresis, PCR-coupled restriction fragment length polymorphism
Discrimination between individual isolates/clonal lineages/ecological interactions within host	'Fingerprinting'—tracking transmission of genotypes/identifying sources of infection and risk factors/competitive interactions	Fingerprinting techniques: e.g., mini/microsatellites, single-strand conformation polymorphism
Genetic markers/linking phenotype and genotype	Identifying phenotypic traits of clinical and epidemiological significance	Genotype linked to phenotype *via*: (i) genetic map; (ii) representation display analysis; (iii) sequencing and/or real-time PCR of genes thought to be linked to phenotypic traits

[a] In some cases, there may be overlap between the tools (regions of DNA) used and requirement. This will depend on the group or organisms being studied and the level of variation detectable by a particular approach.

(e.g., Morgan-Ryan et al., 2002). However, an increasing number of genetically distinct variants has been detected within the species *C. parvum* (see Chalmers et al., 2002; Ong et al., 2002; Siefker et al., 2002; Thompson, 2002b). A number of these genotypes appear to represent distinct species. However, several host species are susceptible to infection with more than one genotype, and their host range and taxonomic status remain to be resolved. Recent molecular and biological evidence has demonstrated that *Cryptosporidium* is more closely related to gregarine protozoa than to coccidia (Carreno et al., 1999; Hijjawi et al., 2002). The recognition of *Cryptosporidium*'s affinities with this group not only helps to explain the increasing numbers of novel genotypes that are being discovered, but also emphasises that the specificity of environmental detection procedures for *Cryptosporidium* could be compromised by "cross-reactivity" with gregarine protozoa ubiquitous in fresh water environments (Bull et al., 1998; Hijjawi et al., 2002; Tenter et al., 2002). Also, tapeworms of the genus *Echinococcus* remain a significant public health problem world-wide. In several regions, there is evidence that aetiological agents of cystic hydatid disease/echinococcosis (CE) are extending their range (Eckert et al., 2001). The taxonomy of *Echinococcus* has been a controversial issue for decades, but the outcome of recent molecular epidemiological

studies has served not only to reinforce the need to revise the taxonomy of *Echinococcus*, but also serves to recognise the contribution of early taxonomists. This is because many of the species described over 50 years ago and subsequently invalidated, have now been shown to be valid as a result of extensive molecular studies throughout the world (reviewed by Thompson and McManus, 2002).

In any epidemiological investigation of an infectious disease, there is a need for sensitive and specific diagnostic procedures for detecting the aetiological agents. PCR-based procedures have proven to have greater sensitivity and specificity than 'conventional' diagnostic approaches reliant on microscopy and/or immuno-detection. For instance, sensitive molecular techniques have been developed for both *Giardia* and *Cryptosporidium* which can also provide information on the genotype or species present, for example, by combining PCR with restriction fragment length polymorphism (RFLP) analysis (Groth and Wetherall, 2000; Amar et al., 2002; Caccio et al., 2002). Such an approach is particularly useful if all genetic variants have been characterised previously by DNA sequencing. If this is not the case, then some variants may not be detected. Although an advantage of such procedures is the ease of interpretation, involving the detection of a small number of bands on an electrophoretic gel, the high sensitivity of most PCR-based procedures can also present problems of interpretation. For example, a recent survey of parasites of domestic cats using microscopy found that 5% were infected with *Giardia*, whereas PCR testing revealed that 80% were 'positive', a level supported by the detection of faecal antigen (McGlade et al., 2002). Such a result raises questions concerning both the clinical and epidemiological significance of such presumably low-level infections with *Giardia* which result in minimal excretion of infective stages.

From an epidemiological perspective, the requirement for molecular tools is that they provide some predictive ability with respect to the aetiology of an infection or disease outbreak and the characteristics of the causative agent(s). Such information is essential for control. An important and unresolved question for many parasites relates to their zoonotic potential. For example, although the World Health Organization has considered *Giardia* to have zoonotic potential for over 20 years, either through direct faecal-oral or waterborne routes of transmission, direct evidence has been lacking (Thompson, 1998). Recent molecular data have shown that pets and livestock may harbour zoonotic genotypes of *G. duodenalis*, as well as genotypes which appear to be host specific (Thompson, 2002a). Thus, current evidence suggests that there are three main cycles of transmission that maintain the parasite in domestic environments, involving humans, livestock and companion animals, as well as wildlife cycles, all of which may impact on public health. However, the frequency of transmission of zoonotic genotypes is not yet known (Thompson, 2002b). Studies of livestock suggest that the public health risk from cattle may be minimal, at least in North America and Australia, since the "livestock genotype" appears to predominate in cattle (O'Handley et al., 2000). Similarly, molecular epidemiological data has yet to incriminate wildlife as the original source of waterborne outbreaks of giardiasis. In contrast, a recent study in communities of Assam in India, where *Giardia* occurs in both humans and their dogs (Traub et al., 2002), has provided the first evidence of zoonotic transmission between dogs and humans, by detecting the same genotype of *Giardia* in both host species, not only in the same village but also in the same household (Traub et al., unpublished).

Fundamental questions about the transmission of parasites will also be resolved increasingly through the application of appropriate molecular tools. For example, the ability to

detect *Toxoplasma* in the tissues of domestic and wild animals is raising questions about the maintenance and transmission of *Toxoplasma* in areas where cats are not abundant. In this respect, the application of PCR-based diagnostic procedures in studies of sheep in Europe and native mammals in Australia (Duncanson et al., 2001; Adams et al., unpublished) suggests that the role of vertical transmission may be more important than previously thought for the maintenance of the parasite in some populations. PCR-based procedures also allow the identification of parasites directly from faecal or environmental samples containing stages which do not possess any distinguishing morphological features. The practical value of this has been demonstrated in some recent studies where parasite eggs in faeces were the only stages available for characterisation. For example, PCR-based procedures enabled the identification of species of hookworm in dogs and feral cats in different endemic foci; the role of dogs as mechanical vectors of *Ascaris* eggs in remote communities was demonstrated; and, a new zoonotic tapeworm, *Hymenolepis microstoma*, in remote Aboriginal communities in Australia was discovered (Adams et al., unpublished; Traub et al., 2002; Macnish et al., 2003).

The growing threats of emerging and exotic parasitic diseases have highlighted the limitations of quarantine barriers and preparedness to respond, and emphasized the need to re-evaluate our capabilities in these areas. A rapid response to such diseases is dependent upon their early detection. Early detection of infectious disease agents is essential, and resources for conventional surveillance activities are time-consuming and expensive. Furthermore, the data provided are often limited, particularly in detecting 'novel' agents and determining vector distribution. Future surveillance activities must be supported by rapid 'field tests', more comprehensive epidemiological data and modelling, all of which will be complemented increasingly by technologies that provide information on environmental factors (Thompson et al., 2004). Thus, genomic and remote sensing technologies, coupled with the Geographical Information System (GIS), will be the mainstay for quarantine surveillance activities in the future. For example, in Australia, PCR-based procedures have been applied to the detection of exotic protozoa, such as *Babesia*, *Leishmania* and *Trypanosoma evansi*. As a result, *Babesia gibsoni* was identified recently for the first time in dogs, and organisms consistent with *Leishmania* in kangaroos (see Muhlnickel et al., 2002; http://www.oie.int/eng/info/hebdo/AIS_12.HTM). Surveillance of *Trypanosoma evansi* is ongoing in Australia, both in northern border regions as well as endemic pre-border areas of south-east Asia (Thompson et al., 2004). The latter illustrates a major problem associated with technology, since applying PCR-based procedures in isolated regions of developing countries is very difficult. Such problems are likely to be overcome, at least in the short term, through the use of innovative sampling and storage techniques which allow the easy transport of samples (taken in the field) to reference laboratories where relevant molecular analyses can be performed.

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Identification and classification within the genus, *Trichinella*, with special emphasis on non-encapsulated species

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Since Richard Owen first described *Trichinella* in 1835, two basic positions have emerged on the organization of the genus: (1) a monospecific genus made up of numerous isolates or subspecies; and (2) a genus comprising eight species with at least one additional genotype (*Trichinella* T6) whose classification remains undetermined (La Rosa et al., 2003). Where each view has an historical precedent, the former is based primarily on infectivity data and host range, and the latter is supported by genetic and biochemical data, cross-breeding experiments, and methodically-analysed biological information. Nonetheless, the classification of this genus remains in flux. A compilation of new and old data suggests that this parasite group comprises two significantly differentiated and reciprocally monophyletic lineages which may be better represented by partitioning the encapsulated and non-encapsulated species into two sister genera.

The recent discovery of additional non-encapsulated isolates of *T. pseudospiralis* and the identification of two new non-encapsulated species, *Trichinella papuae* (Pozio et al., 1999) and *Trichinella zimbabwensis* (Pozio et al., 2002), require us to consider the composition and systematics of the genus *Trichinella*, which also includes a multitude of encapsulated species (i.e., *Trichinella spiralis*, *T. nativa*, *T. britovi*, *T. murrelli* and *T. nelsoni*). Although newborn larvae of both non-encapsulated and encapsulated species are able to penetrate and re-program striated muscle cells as they continue their maturation process to fully developed muscle larvae, only the first-stage larva of encapsulated species induces the muscle cell to synthesize collagen, the major component of the cyst surrounding the developing nurse cell. Importantly, the collagen coat allows encapsulated larvae to survive comparatively longer within the tissue, thereby, maintaining their infectivity for extended periods of time in host carcasses. Such capsule development is unique among nematodes and within the genus *Trichinella*, and is characteristic for species which infect mammalian hosts only. The non-encapsulated species, on the other hand, can infect both mammalian and avian hosts or mammalian and reptilian hosts. Consequently, the formation of a capsule may be a derived character unique to this assemblage which arose from non-encapsulated ancestors.

A number of years ago, Zarlenga et al. (1996) examined the expansion segment V (ESV) within the large subunit ribosomal DNA (lsurDNA) from isolates of *T. pseudospiralis* and found substantial sequence variation. This was the first report suggesting that this species was not genetically homogeneous, as originally believed. Given the extent of the sequence variability, the multiple banding patterns upon PCR amplification of ESV sequences and the lack of strong selection pressures on microsatellite-derived allelic variants, the results were consistent with the theory of a two-step, time-dependent accumulation of rare mutational

events rather than a compilation of episodes involving many single-step variances. The multiple banding patterns from ESV sequences present within individual parasites further suggested the presence of multiple alleles. These data were consistent with the hypothesis that non-encapsulated genotypes were geographically separated for sufficient time frames to permit large block changes in the microsatellite sequences. The absence of microsatellite sequences from the encapsulated species within this region of the lsurDNA (Zarlenga and Dame, 1992) provided additional genetic evidence for a unique evolutionary path for the non-encapsulated genotypes. This was validated with the assignment of two newly identified, non-encapsulated species, namely *T. papuae* and *T. zimbabwensis*. The multiplex PCR test for *Trichinella* genotypes (Zarlenga et al., 1999; Pozio and La Rosa, 2003) demonstrated sufficient variation in the ESV of these geographical isolates and species to allow specific identification and differentiation.

The diversity among isolates heretofore assigned to *T. pseudospiralis* was further investigated by La Rosa et al. (2001) when the analysis was expanded to 11 geographically distinct isolates from Palearctic, Nearctic and Australian regions employing ESV, and mitochondrial-derived lsurDNA and cytochrome oxidase 1 (*cox*1) sequences. This study also included biological and biochemical information. High sequence uniformity among Eurasian isolates, and polymorphism among isolates belonging to distinct zoogeographical regions, were observed. Most importantly, the cross-breeding experiments along with the genetic and biological data offered further support that *T. papuae* warranted recognition as a distinct, non-encapsulated species rather than a genotypic variant of *T. pseudospiralis*.

Recently, a third non-encapsulated species, *T. zimbabwensis*, was identified in Africa where *Trichinella* larvae were detected in 39.5% of farm-raised crocodiles (*Crocodylus niloticus*) in Zimbabwe (Pozio et al., 2002). The morphology of adults and larvae is similar to that of *T. papuae*, though cross-breeding did not yield healthy or abundant numbers of F2. This species has been shown to infect both reptiles and mammals but, unlike *T. pseudospiralis*, it does not have a predilection for avian hosts. The propensity of *T. zimbabwensis* to infect both reptiles and mammals suggests that, although diversification of encapsulated *Trichinella* parasites dates to no earlier than that of their mammalian hosts, the non-encapsulated parasites may represent an older, more biologically diverse parasite assemblage.

Historically, delineation of genotypes has been accomplished by a multitude of techniques, including the use of morphological characters, pathogenicity, relative infectivity levels and host range (Dick, 1983). La Rosa et al. (1992) performed an extensive isoenzyme analysis of parasites then known, and found 1–6 of 27 allozymes examined to be unique to the encapsulated species, and 12 of 27 (45%) to be unique to the non-encapsulated *T. pseudospiralis*. Substantial genetic differentiation was observed upon comparing ESV (Zarlenga et al., 1999) and *cox*1 (Nagano et al., 1999) DNA sequences between encapsulated and non-encapsulated *Trichinella* forms. Gasser et al. (1998) demonstrated also the utility of PCR-coupled single strand conformation polymorphism (SSCP) analysis to differentiate *Trichinella* species. At the time these data were generated, however, only one species of non-encapsulated *Trichinella* had been identified.

With the delineation of biological and biochemical characters among geographical isolates of *T. pseudospiralis*, the classification of two additional non-encapsulated species of

Trichinella, and the ever expanding database of molecular and biochemical information on this parasite group, evidence is mounting that the non-encapsulated species constitute a phylogenetically diverse group which is distinct from the capsule-forming species of *Trichinella*. This separation has been further supported by recent study of DNA sequence data, collectively analyzing each of the eight species along with the "outlying" T6 genotype using parsimony and maximum likelihood methods (unpublished). Results consistently group the non-encapsulated species to the exclusion of the encapsulated species, without consideration given to obvious biological or morphological differences. Thus, amidst similarities in the overall life cycles, classification of the encapsulated and non-encapsulated parasites as sister genera remains consistent with the information currently available. To this end, it is our contention that the genus *Trichinella* be subdivided into sister genera, whereby the encapsulated species, *T. spiralis*, *T. nativa*, *T. britovi*, *T. murrelli*, *T. nelsoni* and the undefined *Trichinella* T6 genotype be grouped separately from the non-encapsulated species *T. pseudospiralis*, *T. papuae* and *T. zimbabwensis*. The first such suggestion made by Pozio et al. (2001) recommended that additional genetic markers be evaluated. To date, other than historical opinion, no data are available which refute a 'bifurcation' of the genus.

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Filarial nematodes and *Wolbachia*: a veterinary perspective

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Most filarial species of veterinary and medical importance, including *Dirofilaria immitis*, *D. repens*, *Onchocerca* spp., *Brugia* spp. and *Wuchereria bancrofti*, have been shown to harbour intracellular bacteria of the genus *Wolbachia* (reviewed by Bandi et al., 2001). These bacteria, which belong to the Rickettsiales, are believed not to be infectious to the vertebrate hosts of the filariae, but have been implicated in the immunopathogenesis of filarial diseases (Taylor et al., 2000; Bandi et al., 2001). They also appear to be required by the nematode for normal development and reproduction, and could represent a target for the control of filariasis (Bandi et al., 2001; Hoerauf et al., 2002). Moreover, the specificity of the immune response against *Wolbachia* could provide for sero-diagnostic applications (Simon et al., 2003). The identification of *Wolbachia* in filarial nematodes has stimulated studies of the phylogeny of this nematode group using molecular data sets, which could also be useful for the development of diagnostic tools (Casiraghi et al., 2001).

Symbiosis between *Wolbachia* and filarial nematodes is supported by the following observations: (1) In filarial species which are positive for *Wolbachia*, all individuals examined harbour this bacterium, (2) There is support for a *Wolbachia*-nematode co-evolution, and (3) *Wolbachia* is vertically transmitted to the nematode offspring, with no evidence of horizontal transmission (Bandi et al., 2001). This information provides some clues about the relationship between *Wolbachia* and filarial nematodes, and suggests that this association is obligatory and perhaps beneficial to the nematode, i.e. there is 100% prevalence in infected species, there is phylogenetic evidence for a long co-evolutionary history, and the strict vertical transmission may promote the evolution of mutual interactions (see Yamamura, 1993). The results of experiments using antibiotics are consistent with this information. Indeed, antibiotic treatments (e.g., using tetracycline and derivates) against *Wolbachia* are deleterious to the nematode hosts, causing, in most cases, developmental arrest, permanent disruption of reproduction, and interference with motility and long-term survival of the nematode (Bandi et al., 2001; Casiraghi et al., 2002; Hoerauf et al., 2002).

The effects of tetracycline and other antibiotics on filarial nematodes have implications for the control of filarial diseases. These effects are evident particularly in human onchocerciasis. Death and degeneration of microfilariae which migrate into the eyes are responsible for the inflammatory effects, resulting in ocular damage and impaired vision. Long-term treatment with doxicycline results in the sterilization of adult parasites and, consequently, leads to a reduction in the number of microfilariae undergoing ocular migration (Hoerauf et al., 2002). In the case of *D. immitis*, treatment with tetracycline or its deriva-

tives also leads to worm sterility. However, this effect is of limited value for the control of heartworm disease in dogs and cats, given the efficacy of current treatment regimens. Thus, prolonged or intermittent, long-term treatment with antibiotics should be investigated for effects against adult parasites, such as those observed in the bovine parasite *Onchocerca ochengi* (see Langworthy et al., 2000).

The role of *Wolbachia* in the immunity against filarioids has several implications, from a veterinary perspective. Extracts of filarial nematodes harbouring *Wolbachia* have been shown to stimulate innate responses in monocytes via the expression of specific receptors and the production of nitric oxide and various cytokines (Brattig et al., 2000; Taylor et al., 2000). These effects are thought to be associated with *Wolbachia*, particularly those derived from lipopolysaccharide (LPS)-like molecules and bacterial proteins (Brattig et al., 2000; Taylor et al., 2000; Bandi et al., 2001). As expected for bacterium-derived antigens, there is evidence that *Wolbachia*-induced immune profiles are indicative of a Th1-type response (Marcos-Atxutegi et al., 2003). Recently, the *Wolbachia* surface protein (WSP) has been shown to induce chemokinesis in dog neutrophils, with the production of interleukin-8 (IL-8) (Bazzocchi et al., 2003). This surface protein also induces a specific antibody response in humans and animals infected with filarial nematodes (Bazzocchi et al., 2000; Simon et al., 2003).

The antibody response against WSP may also have implications for the diagnosis of pulmonary dirofilariasis in humans. Indeed, people living in areas endemic for dog heartworm, while being clinically healthy, are frequently seropositive for *D. immitis*. This makes it difficult to distinguish between healthy humans in endemic areas and those patients with pulmonary nodules due to *D. immitis*, using a serological assay based upon nematode antigens. However, given the association between *Wolbachia* and *D. immitis*, this distinction appears to be possible through the titration of anti-WSP antibodies (see Simon et al., 2003).

Studies implicating an immunological role for *Wolbachia*-associated molecules stimulate a re-evaluation of the pathogenesis of heartworm disease. Alterations of blood vessels along with the inflammatory responses surrounding both dying worms and those present in the lungs and kidneys could be attributed, in part, to *Wolbachia*. In addition, the shock-like side effects of microfilaricidal treatments may be associated with the release of *Wolbachia*-associated molecules from dying larvae. Although the relationship between *Wolbachia* and the effects of microfilaricidal treatments has not yet been demonstrated in dogs, there is evidence from research in humans to support this hypothesis (Taylor et al., 2000; Keiser et al., 2002).

Studies aimed at developing vaccines against filarial nematodes could also focus on the bacterial symbionts and, in so doing, address the following questions: (1) Does *Wolbachia* play a role in polarizing and/or modulating the immune response in filariasis? (2) Does immunization with extracts from filarial nematodes not harbouring *Wolbachia* produce different immune response patterns compared with those containing *Wohlbachia*? and (3) Can information be gleaned through investigating the immune responses against *Wolbachia* in dogs protected against heartworm infection compared with those in which immunization was not successful? Polymorphism in the receptors of immune-related molecules involved in host responses against bacterial molecules should also be investigated in such dogs.

In conclusion, the identification of *Wolbachia* in filarial nematodes has provided new insights and stimuli for future studies, with significant implications in the areas of systematics, diagnosis, immunology and pathogenesis in both the medical and veterinary fields. Phylogenetic studies of filarial nematodes are leading to an improved knowledge of their association with *Wolbachia* and an understanding of the diseases they cause. Analysis of mitochondrial gene sequence data has recently provided evidence for a relatively close relationship between the genera *Dirofilaria* and *Onchocerca*, which contradicts their taxonomic assignment to different subfamilies, the Dirofilariinae and Onchocercinae (Casiraghi et al., 2001). Over time, new data should assist in answering these and other complex questions regarding this fascinating group of organisms.

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Some recent advances in mitochondrial genomics of parasitic nematodes—implications for molecular systematic and population genetic investigations

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Many species of parasitic nematodes are pathogens of plants and/or animals, including humans, causing significant diseases and major socio-economic losses. Central to the control of these parasites is knowledge of their population genetics, which can also have important implications for understanding ecology, transmission patterns and the development of drug resistance (Anderson et al., 1998; Blouin, 1998; Viney, 1998; Gasser and Newton, 2000). The basis for investigating population genetics is the accurate analysis of genetic variation, which is generally considered to be widespread in parasite populations (e.g., Grant, 1994; Anderson et al., 1998; Blouin, 1998; Viney, 1998). Molecular methods have proven useful for assessing genetic variation within and among parasite populations (Nadler, 1990; Grant, 1994; Gasser and Newton, 2000). Mitochondrial (mt) DNA markers have been considered to be applicable to population genetics and systematic investigations due to their high mutation rates and proposed maternal inheritance (Avise et al., 1987; Avise, 1991, 1994, 1998; Anderson et al., 1998; Blouin, 1998, 2002). While there is a wealth of information on the mt genomes for a range of animals other than helminths, there is a paucity of knowledge for parasitic nematodes of socio-economic importance. For example, the orders Strongylida, Ascaridida, Spirurida and Rhabditida contain many species infecting animals and/or humans (Anderson, 2000), but until recently, only a small number of complete mt genome sequences was available for species from these orders (reviewed by Hu et al., 2003d). The purpose of this presentation was: (1) to provide an account of some of the approaches used for mt genome sequencing and for population genetic studies; (2) to describe some applications of mt gene markers to study the molecular systematics and population genetics of parasitic nematodes; and (3) to conclude by emphasizing the prospects and opportunities provided by recent studies of the mt genomes of parasitic nematodes.

Recently, Hu et al. (2002a) evaluated a simple long PCR for the amplification of the entire mt genome (in two overlapping fragments of ~5–10 kb each), from 10% of the total genomic DNA isolated from single adults of each of the two species of human hookworm, *Ancylostoma duodenale* and *Necator americanus* (Strongylida). Then, 12 other species of secernentean nematode from the orders Strongylida, Ascaridida and Rhabditida were tested (Hu et al., 2002a). The primer sets 39F–42R and 5F–40R (see Hu et al., 2002a) used for human hookworms could also achieve specific amplification of the ~5 kb and ~10 kb fragments from four species of ascaridoid nematode, whereas the reverse primers in the *nad*1 and *rrn*L gene had to be re-positioned (374 bases downstream and 414 bases upstream, respectively) to achieve effective amplification from the equine ascaridoid (*Parascaris equorum*), bovine and porcine lungworms (*Dictyocaulus viviparus* and *Metastrongylus pudendotectus*, respectively), the large strongyle (*Strongylus vulgaris*) from the horse or from the barber's pole worm (*Haemonchus contortus*) of sheep. Interestingly, the two amplicons produced for *Strongyloides stercoralis* were the opposite in size to those amplified from the two human

hookworms and other nematodes (Hu et al., 2002a), which indicated that the mt gene arrangement for *S. stercoralis* was distinctly different from the other 13 parasitic nematodes examined, which was later confirmed by sequencing its complete genome (Hu et al., 2003a).

Using the long PCR approach (Hu et al., 2002a), the complete mt genome sequences were determined for the human hookworms, *N. americanus* and *An. duodenale*, the canine heartworm *Dirofilaria immitis*, and *S. stercoralis*. Comparative analyses of current data for nematodes revealed that the mt genomes of *An. duodenale*, *N. americanus*, *Ascaris suum* and *Caenorhabditis elegans* all had the same gene arrangement (GA2), as did *O. volvulus* and *D. immitis* (GA3) (see Keddie et al., 1998; Hu et al., 2002b, 2003b), which were, interestingly, all distinctly different from that of *S. stercoralis* (GA1) and from *T. spiralis* (GA4) (Hu et al., 2003a). Between arrangements GA1 and GA2, there were two shared gene boundaries (*atp*6–*nad*2 and *cox*2–*rrn*L); irrespective of the different positions of the *trn* genes, there were 11 gene- or gene block- translocations. Comparison between arrangements GA1 and GA3 revealed one shared gene boundary (*cox*2–*rrn*L) and 11 gene- or gene block-translocations. Arrangement GA1 was also very distinct from that of *T. spiralis* (GA4), the latter of which was very different from the other nematodes examined and, interestingly, was more similar to those of coelomate metazoans (cf. Lavrov and Brown, 2001). Between arrangements GA1 and GA4, there was one shared gene boundary (*nad*5–*nad*4), 7 translocations and 4 inversions. Unique to arrangement GA4 was the presence of a putative *atp*8 gene which was absent from the mt genomes with arrangements GA1–GA3. These findings indicate clearly that the extent of mt genome rearrangement within the Nematoda is significantly higher than for mt genomes of other animal groups, such as most arthropods (Shao et al., 2001), platyhelminths (Le et al., 2002a) and vertebrates (Boore, 1999), which should have implications for elucidating molecular mechanisms leading to these gene rearrangements and for studying the evolutionary history of nematodes.

The availability of complete mt genome sequences provides a rich resource for selecting appropriate genetic markers for molecular systematics and population genetics studies of parasitic helminths (reviewed by Hu et al., 2003d). For example, using single-strand conformation polymorphism (SSCP) analysis, Hu et al. (2002c) examined the genetic structures of hookworm populations, in order to establish degrees of haplotypic diversity and population substructuring within some species. Sequence heterogeneity was studied in a portion of the cytochrome *c* oxidase subunit 1 gene (designated p*cox*1) for *An. duodenale* from China, *Ancylostoma caninum* from Australia, and *N. americanus* from China and Togo by using SSCP combined with selective DNA sequencing. The p*cox*1 sequences were characterised for individual nematodes displaying genetic variation within each of the three species, and those were compared with p*cox*1 sequences of four other species of hookworm. While intraspecific variation in the p*cox*1 sequence ranged from 0.3 to 3.3% for *An. duodenale*, ~0.5–9% for *An. caninum* and 0.3–4.3% for *N. americanus*, interspecific differences varied from ~5 to 13%. The sequence data also provided information on nucleotide compositions and substitution patterns. Genetically distinct groups were detected within *An. caninum* and *An. duodenale*, revealing significant population substructuring within these species. Given that *An. caninum* is known to infect and cause eosinophilic enteritis in humans in Australia (Prociv and Croese, 1996; Beveridge, 2002), it is tempting to speculate that the different genetic variants (haplotypes) of this hookworm have differing affiliations to the dog, cat and human hosts and that (at least) one of these is zoonotic; this proposal should be tested. Also, all *N. americanus*

individuals from China differed from those from Togo at four nucleotide positions, supporting a previous proposal (based on ribosomal DNA sequence data; Romstad et al., 1998) that *N. americanus* may represent a species complex. This question has been addressed further in a more recent study (Hu et al., 2003c). Therein, the mt genome sequence from a *N. americanus* individual from Togo was compared with another from China, to provide an estimate of the magnitude of genetic variability. The comparison revealed sequence differences of 3–7 and 1–7% (nucleotide and amino acid levels, respectively) in the 12 protein-coding genes. The most conserved of these was the *nad*4L gene, whereas the *nad*1 gene was the least conserved. Nucleotide differences were also detected in 14 of the 22 *trn* genes (∼1–13%), the AT-rich region (∼8%), the non-coding regions (8–25%), and in the two small (*rrn*S) and large (*rrn*L) subunits of mt *rrn* genes (∼1%). Comparison of the *rrn*L sequences from multiple individuals revealed nine unequivocal differences between *N. americanus* from the two countries. Overall, these findings revealed substantial genetic variation in *N. americanus* and supported the "cryptic species concept", although biological evidence would be useful to further support the molecular data. Irrespective of species status, the detection of unexpectedly high levels of mt genetic variation within *N. americanus* may have implications for the transmission and control of necatoriasis, because it is possible that each different gene pool of *N. americanus* possesses different biological, ecological and disease characteristics.

Extending the application of the SSCP-sequencing approach to another parasite group, Hu et al. (2002d) also investigated the population genetic structure of the bovine lungworm, *Dictyocaulus viviparus*, in southern Sweden. This parasite belongs to the same order (Strongylida) as hookworms, but is placed in the superfamily Trichostrongyloidea. It also has a direct life cycle, and the adult worms occur in the bronchi and trachea of bovids and causes bronchitis, commonly called "husk" (Allan and Johnson, 1960). Husk has been reported to be endemic in many countries, such as Britain, The Netherlands (Eysker and van Miltenburg, 1988), France (Tessier and Dorchies, 1997), Belgium (Vercruysse et al., 1998), Sweden (Höglund et al., 2001), the USA (Eddi et al., 1989) and Tanzania (Thamsborg et al., 1998), and causes substantial economic losses to the farming industry (Corwin, 1997; Woolley, 1997). Hu et al. (2002d) subjected *D. viviparus* individuals (n = 252, collected from cattle representing 17 farms in Sweden) to SSCP analysis of p*cox*1. Samples with distinct SSCP profiles were then sequenced. In total, 12 distinct p*cox*1 haplotypes (393 bp) were defined for the 252 individuals, and pairwise sequence differences among the haplotypes ranged from 0.3 to 2.3%. Average haplotype diversity and nucleotide diversity values were 0.16 and 0.002, respectively, and there was no particular correlation between p*cox*1 haplotypes and their geographical origin. The "overall fixation" values were 0.77 (F_{ST}) and 0.65 (N_{ST}). The results showed that both the mt DNA diversity within populations of *D. viviparus* and the gene flow among populations were low. Interestingly, this is similar to findings for some parasitic nematodes of plants and of insects (e.g., Hugall et al., 1994; Blouin et al., 1999), but distinctly different from gastrointestinal trichostrongyloid nematodes of domesticated ruminants with relatively high levels of diversity and gene flow (Blouin et al., 1992, 1995; Blouin, 2002). This difference was interpreted to relate mainly to differences in host movement as well as parasite biology, population sizes and/or transmission patterns, and should thus be of epidemiological significance.

In conclusion, there is still a paucity of information on structural and functional aspects of mt genomes and on the population genetics of parasitic nematodes. Circumventing the

technical limitations of sequencing such genomes from individual nematodes via the use of a long PCR-based approach provides the prospect of rapidly studying the mt genomes from a wide range of species representing different taxonomic groups. Recent studies have provided some new insights into mt genome structures, and the population genetics, taxonomy and evolution of parasitic nematodes of socio-economic importance. The molecular tools available and advances made provide unique prospects and opportunities for future work on a number of fundamental areas, such as the mechanisms and processes of gene rearrangements, mutation rates, the inheritance of mt genomes and genes, and the systematics and population genetics of nematodes. This also provides a foundation for tackling questions regarding the ecology and epidemiology of parasitic nematodes, thus contributing, in the broader sense, to the diagnosis and control of parasitic diseases.

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Progress in the molecular diagnosis of cyathostomins—implications and prospects

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The subfamily Cyathostominae represents a complex group of nematodes which is ubiquitous in horses. Within this group are more than 50 species (Lichtenfels et al., 2002), the basic biology of which is largely unknown. These parasites have direct life cycles, in which they undergo a period of inhibited development as early third stage larvae (EL3s) in the large intestinal wall. These EL3s play a central role in the pathogenesis of cyathostomin infections, as large numbers can accumulate and subsequently become reactivated to cause a syndrome known as larval cyathostominosis (Ogbourne, 1976).

Most infected horses have tens of thousands of these parasites without developing clinical disease. However, in some animals a severe inflammatory colitis develops which is associated with the presence of large numbers of reactivating larvae (Giles et al., 1985; Love et al., 1999). The principal clinical effect of this syndrome is weight loss, but horses can also develop diarrhoea, subcutaneous oedema and/or pyrexia (Love et al., 1999). Cyathostomins have also been associated with various types of equine colic, attributable to the presence of large numbers of mucosal larvae (Uhlinger, 1990; Mair and Pearson, 1995; Murphy and Love, 1997; Mair et al., 2000). Inhibited cyathostomins have limited susceptibility to several of the currently available anthelmintics (Eysker et al., 1992; Klei et al., 1993; Proudman and Matthews, 2000). This is compounded by the fact that, unlike members of the Strongylinae, anthelmintic resistance is common in cyathostomins, particularly with regard to benzimidazoles (Kaplan, 2002).

Despite their clinical importance, little is known about the biology of cyathostomins which makes it difficult for rational treatment and control strategies to be devised. The detailed biology of the pre-parasitic and parasitic stages is not yet known, and the contribution that individual species make to the pathogenesis of mixed infections is unclear. To some extent, this relates to the fact that egg and larval stages of these nematodes cannot be identified to species using morphological criteria. Traditionally, identification has relied on the morphological examination of adult parasites (Lichtenfels, 1975). Prevalence studies have indicated that, although many species were often present, 90% of the parasites present belonged to a limited number of species (Ogbourne, 1976; Mfitilodze and Hutchinson, 1990; Bucknell et al., 1995; Gawor, 1995). As such studies required access to intestinal contents of horses and only provide information at a single time point, non-invasive methods for species identification have been required to study the biology and epidemiology of these parasites further. To this end, experiments were undertaken to develop molecular probes for cyathostomin species.

To provide sequence data from which specific DNA probes could be designed, the intergenic spacer (IGS) region was amplified by PCR and sequenced from 16 species of cyathostomin (Kaye et al., 1998). As the level of sequence conservation within the IGS region amongst species was relatively high, species-specific oligonucleotide probes were designed from short sequences (19–21 nucleotides). Probes were designed for the following common species: *Cylicocyclus ashworthi*, *Cylicocyclus nassatus*, *Cylicocyclus insignis*, *Cyathostomum catinatum*, *Cylicostephanus goldi* and *Cylicostephanus longibursatus* (see Hodgkinson et al., 2001). These probes were initially tested for their specificity by hybridisation against the IGS PCR products from DNA from 19 heterologous (morphologically identified) species (Hodgkinson et al., 2001; Hodgkinson et al., 2003). The species against which the probes were tested were found to be common in previous prevalence studies (e.g., Ogbourne, 1976). Furthermore, they represented species

with the greatest sequence similarity in the IGS to the particular probes being validated. Due to the small size of the probes, the hybridisation step was performed at a relatively low temperature (32–37 °C), and subsequent washing was performed at high stringency. Each probe proved to be specific to the homologous species, and the experiments indicated that the probes could discriminate between species, with as little as one base-pair mismatch. Following validation of specificity, each probe was shown to be able to identify the species present in samples representing eggs, third stage larvae (L3s) and fourth stage larvae (L4s). A Southern blot approach was used initially, but was then replaced by a PCR-based enzyme-linked immunosorbent assay (PCR–ELISA) system (Hodgkinson et al., 2003). This system utilizes biotinylated species-specific probes to immobilise digoxigenin (DIG)-labeled IGS–PCR products of the homologous species on to the wells of a streptavidin-coated ELISA plate. Detection is performed by incubating the products with an alkaline phosphatase-conjugated anti-DIG antibody, followed by the addition of an alkaline phosphatase substrate.

Once the PCR–ELISA system was established, the oligonucleotide probes were used to identify the species of larvae present in the diarrhoeic faeces from horses with larval cyathostominosis (Hodgkinson et al., 2003). These experiments were performed because little is known about the pathogenesis of this syndrome and because various hypotheses had been formulated to explain why some horses develop severe colitis associated with larval reactivation. It had been suggested that particular species, for example, *Cc. insignis*, are more pathogenic than other species, this theory being based on the observation that large larvae representative of this species were often found in the diarrhoeic faeces of affected horses (Reinemeyer and Powell, 1986). Variation in the clinical signs had also been attributed to the number of larvae in the intestinal wall and/or the characteristics of the individual host's inflammatory or immune response (Mair, 1994). No information had been published to provide support for any of these theories. There had been attempts to morphologically identify L4s in diarrhoeic faeces from clinical cases, however, discriminating features of the L4s were found to be limited (e.g., Reinemeyer and Powell, 1986; Chiejina and Mason, 1997). Thus, the oligonucleotide probes were used to identify the species of L4s present in diarrhoeic faeces from 17 horses with clinical larval cyathostominosis. In our study, *Cs. longibursatus* and *Cc. nassatus* were shown to be the commonest species. In contrast to previous findings (e.g., Ogbourne, 1976), L4s of *Cy. catinatum* were found in only a small number of horses ($n = 5$) and represented 1.7% of the total population analysed in the DNA hybridisation studies (Hodgkinson et al., 2003). This result may have reflected geographical or seasonal variation in the prevalence of this particular species. Interestingly, *Cc. insignis* was not detected using the respective probe, which did not support the previous assumption that this species was particularly pathogenic. *Cs. goldi* was shown to be present in a number of affected horses, but in smaller numbers than both *Cs. longibursatus* and *Cc. nassatus*, which is consistent with previous studies by other workers (Ogbourne, 1976; Bucknell et al., 1995). Of the 546 L4s collected, PCR products from 24 parasites did not hybridise to the positive control probe or to any of the species-specific probes. Thus, these L4s may have represented species of cyathostomin which had not been encountered previously. Analysis of the molecular data indicated that there was no association between the species identified and the outcome of disease or severity of diarrhoea. The data supported the proposal that several common species of cyathostomins play a role in clinical disease, and they did not

suggest a primary role for a single species of cyathostomin. Nonetheless, it is possible that a single species could be involved in the initial triggering of larval reactivation.

The diagnostic oligonucleotide probes developed were subsequently used to identify the species of eggs present in faecal samples from 12 horses prior to and following anthelmintic treatment (K.L. Freeman et al., unpublished data). Of the species for which probes were designed (see above), eggs of *Cs. longibursatus*, *Cy. catinatum*, *Cs. goldi* and *Cc. nassatus* were detected prior to treatment. These species were detected at a similar frequency in the faecal samples tested from individual horses. Eggs of *Cc. ashworthi* and *Cc. insignis* were not identified prior to treatment. Following treatment with pyrantel and five daily treatments of fenbendazole at recommended dose rates, the egg reappearance period was at least 28 days. In each horse, only one of the six species was present at the first time point examined after treatment. In these samples, the faecal egg counts were low (25–125 eggs per gram), which may have affected the number of species identified per sample. The species present at the first parasite-positive sample varied amongst horses, with the most common species detected being *Cs. longibursatus* or *Cy. catinatum*. As the faecal egg counts increased over time, the number of species identified in each sample increased. Eggs of *Cc. insignis* and *Cc. ashworthi* (not detected prior to treatment) were identified in samples taken later in the post-treatment period. The parasites in this population were resistant to benzimidazoles (as indicated by a faecal egg count reduction test), which may therefore indicate that the presence of resistance is not associated with a particular species.

Overall, the results from these recent studies emphasise the utility of a PCR-based approach for investigating the biology of cyathostomins. The fact that this approach identifies species at all stages in their development allows the design of experiments which have not been attempted previously. The sensitivity and specificity of the molecular approaches will allow the detailed study of the role(s) of these common species in the pathogenesis and epidemiology of cyathostominosis.

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Conceptual advances in the evolutionary history of tissue–cyst forming coccidia of veterinary importance

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New genetic information is changing our understanding of the composition, systematics and epidemiology of tissue cyst-forming coccidia, a diverse but poorly characterised group of protozoan parasites. Of the hundreds of described species within the Sarcocystidae, assigned to the genera *Sarcocystis*, *Toxoplasma*, *Besnoitia*, *Hammondia*, *Frenkelia*, *Isospora* and *Neospora*, complete life-history descriptions are available for only a few. Although ultrastructural characteristics provide those with expertise the means to differentiate many of the named species, we lack a thorough comparative context which would promote a more comprehensive understanding of the biodiversity of this parasite group, and the epizootiology of its constituent taxa.

Genetic information, shared by all developmental stages of a parasite, may serve as a powerful means to identify individual taxa and reconstruct the descent relationships among related parasite isolates. Comparative analyses of ribosomal DNAs, plastid-encoded genes, anonymous DNA fragments and microsatellite data have recently elucidated features of the population genetics and molecular systematics of this important parasite group. Such

data have been used to address the earliest evolutionary history of the group, such as the origins of tissue cyst parasitism, and recent events exemplified by the world-wide advance of particular *Toxoplasma gondii* genotypes. Undoubtedly, more surprises lay ahead as the tools of molecular systematics and population genetics are applied to these pervasive, but poorly understood, protistan parasites.

The earliest coccidia apparently employed direct faecal-oral transmission, as do the most extensively differentiated, basal extant members of the group (Carreno and Barta, 1999; Slapeta et al., 2003). Sexual reproduction of such parasites in the gastrointestinal tract of the host culminates in the excretion of oocysts which, in turn, leads to infection upon ingestion by a new, susceptible host. Although also characterised by these general coccidian features, the species of the genus *Cryptosporidium* do not share specific common ancestry with other coccidian genera. Instead, they relate most closely to gregarine parasites of arthropods (Carreno et al., 1999). This proposal helps explain their insensitivity to anti-coccidial drugs and their distinction from coccidia in several morphological, life-history and physiological traits. This example emphasizes how defining eukaryotic microbial biodiversity and systematics can establish a context for more applied biological research.

Within the true coccidia, subsequent adaptation enabled predation to be exploited for transmission. These "tissue–cyst forming coccidia" encyst in the extra-intestinal musculature of susceptible herbivores upon ingesting oocysts excreted by their predators, in whom the sexual reproductive phase occurs. To date, the best studied examples employ feline and canine definitive hosts, which have independently given rise to distinct parasite lineages; some of the parasites employing carnivorous marsupials definitive hosts have begun to be characterized, spurred by the recognition of opossums (*Didelphis virginiana*) as reservoirs for *Sarcocystis neurona*, the agent of equine protozoal myeloencephalitis (Shaw and Lainson, 1969; Dubey and Lindsay, 1999; Tanhauser et al., 1999; Dubey et al., 2000; Cheadle, 2001; Dubey et al., 2001a,b; Rosenthal et al., 2001; Stanek et al., 2002).

Reversion to a direct life cycle has occurred in some parasites no longer encysting in the tissue of intermediate herbivorous hosts, as their ancestors once did. Thus, the genus *Isospora* mistakenly includes both these "secondarily homoxenous" members, as well as a second group of parasites whose progenitors were not transmitted through carnivorism (Carreno and Barta, 1999). Various means have been proposed to revise the systematic classification, so that a unique name designates each of these evolutionarily distinct groups (Barta et al., 2001; Modry, 2002; Upton, 2002; Su et al., 2003).

In another departure from the ancestral pattern, wherein consumption of tissue cysts initiates the sexual stage in the carnivore gut, ingestion of *T. gondii* tissue cysts can induce formation of such cysts directly. The ability to circumvent the sexual stage entirely constitutes a fundamental departure from the typical life history of these parasites (Su et al., 2003), and may help explain the global prevalence of merely a handful of *T. gondii* non-recombinant genotypes (Grigg et al., 2001; Durand et al., 2003). It is unknown how frequently such clonal propagation may occur in other tissue cyst-forming coccidia. Simply put, we need to understand whether we are confronted with discrete species comprising an array of interbreeding individuals or whether, instead, myriad genetically isolated and phylogenetically independent clones characterize this parasite fauna. Answering this question will require us to ascertain how allelic variation at multiple genetic loci is partitioned over space and through time (cf. Tibayrenc et al., 1991; Tibayrenc and Ayala, 2002).

Initial investigations into species of *Besnoitia* and *Sarcocystis* suggest that taxa defined by subtle morphological or life-history characteristics may lack intraspecific genetic polymorphism, and that such taxa may be consistently distinguished from one another by subtle differences at several genetic loci (Tanhauser et al., 1999; Dubey et al., 2001b; Rosenthal et al., 2001). The exchange of genetic information would therefore appear to occur infrequently, if ever, among such taxa, promoting their evolutionary diversification. However, the absence of substantial interspecific differentiation at more conserved genetic loci suggests a relatively recent origin for many such tissue–cyst forming coccidia, perhaps reflecting recent radiations of lineages playing specialized ecological and epidemiological roles.

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